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Porous cellulose as promoter of oil production by the oleaginous yeast *Lipomyces starkeyi* using mixed agroindustrial wastes

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ABSTRACT

Enhanced single cell oil (SCO) production by the oleaginous yeast *Lipomyces starkeyi* DSM 70296, immobilised on delignified porous cellulose, is reported. Pure glucose media were initially used. The effects of substrate pH and treatment temperature were evaluated, showing that 30 ◦C and pH 5.0 were the optimum conditions for SCO production by the immobilised yeast. The immobilisation technique led to increased lipid accumulation and cell growth by 44% and 8%, respectively, in the glucose media, compared to free cells in suspension. This positive effect was also shown when low concentration mixed agro-industrial waste suspensions were used as substrates, leading to 85% enhanced SCO production in comparison with free cells. Higher fatty acid (HFA) analysis showed that yeast immobilization led to increased formation of unsaturated HFAs (6%) and reduced saturated HFAs (5%) compared to free cells.

Keywords: Single cell oil, *Lipomyces starkeyi*, cellulose, immobilisation, higher fatty acids

1. Introduction

The use of first generation biodiesel (mainly consisting of fatty acid methyl esters produced from crop oils) led to increase of the cost of raw materials, since oilseed crops require energy and arable land competing with food and feed production. Therefore, it has become imperative that biofuel production should depend on renewable resources such as non-food crops and agroindustrial wastes (AIW) and by-products. Recent research efforts have also focused on biodiesel production using microbial oil (single cell oil; SCO) that accumulates in oleaginous microorganisms, which can grow on various resources such as AIW (Koutinas et al., 2014; Tsouko et al., 2016). The bioconversion of AIW into SCO can lead to decrease of biodiesel production cost with simultaneous recycling and valorisation of these wastes.

For this purpose, a variety of oleaginous microorganisms have been proposed (Leiva-Candia et al., 2014). Among them, *Lipomyces starkeyi,* an oleaginous yeast isolated from soil, has a lipid accumulation ability of about 65% of its dry cell weight when grown in synthetic sugar media (Anschau et al., 2014; Lin et al., 2011). It is capable of utilizing mixed sugars, including glucose, xylose, mannose, and cellobiose, and can degrade extracellular polysaccharides by secreting glycosidases (Calvey et al., 2016; Zhao et al., 2008). Efforts to produce SCO from AIW using *L. starkeyi* faced lower lipid accumulation productivities compared to those obtained in synthetic media, thus limiting its potential industrial applications (Ali El-Naggar et al., 2011; Yu et al., 2011).

The use of cell immobilization techniques in fermentation processes has offered numerous advantages compared to conventional free cell or enzyme systems, including improved productivities and product quality, feasibility of continuous

processing, protection against physicochemical and shearing stresses, etc., (Kourkoutas et al., 2004; Liu & Chen, 2016). Various types of materials have been proposed as carriers for cell or enzyme immobilisation, including cellulosic AIW. Among these, porous delignified wood cellulose (DC) was proved to be a significant promoter of the fermentation activity and viability of various microorganisms when used as immobilisation carrier. DCs produced by lignin removal of various plant lignocellulosics (wood, straw, husk, etc.) were shown to have a complex porous structure, including pores and tubes with sizes down to micro and nano scales (Ganatsios et al., 2014; Koutinas et al., 2012; Kumar et al., 2014).

Regarding the use of AIW for the production of valuable products, the bioconversion of mixed substrates has several advantages such as: (i) reduction of transportation and disposal costs; (ii) possibility to develop complete fermentation feedstocks without need for nutrients addition; (iii) ability to exploit AIW from low-capacity production plants by supplying them to a central treatment plant; (iv) improvement of the nutritional value of livestock feeds; (v) production of various valuable products (Aggelopoulos et al., 2013; 2014;). Specifically, the organic load of AIW can be recovered or hydrolyzed (chemically or biologically) to produce glucose, amino acids and phosphate, which can then be (bio)transformed into valuable products such as carbon and nitrogen sources, alcohols (ethanol, butanol, 2,3-butanediol), organic acids (citric, succinic, lactic, erucic, acrylic, adipic, etc.), proteins, enzymes, oils, lignin, phytochemicals, glycerol, phytosterols, antioxidants, flavonoids, carotenoids, biodegradable plastics, aroma compounds, esters, biosurfactants, prebiotic oligosaccharides, etc., (Aggelopoulos et al., 2014; Andersen et al., 2015; Leiva-Candia et al., 2014; Pleissner et al., 2016; Sharma et al., 2016).

Mixed AIW substrates containing cheese whey, molasses, orange and potato

pulp, were recently used for growth of various species (*Kluyveromyces marxianus*, kefir, and *Saccharomyces cerevisiae*), showing a promotional effect of orange pulp when added in the growth media (Aggelopoulos et al., 2013; 2014). Molasses on the other hand, is a carbohydrate-rich by-product of the sugar production industry (45-55% fermentable sugar; mainly sucrose), which is commonly used for bioethanol and baker's yeast production worldwide. It has also been extensively used for numerous other valuable fermentation products such as SCO (Sharma et al., 2016; Vieira et al., 2016), alone or mixed with other types of substrates (Andersen et al., 2015).

Based on the above studies, the aim of the present investigation was to evaluate the growth and SCO accumulation ability of the oleaginous yeast *L. starkeyi*, immobilised on DC, using low-sugar AIW mixtures consisting of molasses and orange pulp as substrates.

2. Materials and methods

2.1 Microorganism and media

The strain *L. starkeyi* DSM 70296 was preserved at 4 °C in 2% agar slopes containing 10 g L^{-1} yeast extract, peptone, and D-glucose (Tsakona et al., 2014). The same medium, without the addition of agar, was used for the preparation of inocula, at 30 ^{o}C. The yeast was then cultivated, at 30 ^{o}C, in synthetic glucose media containing (g L^{-1}): glucose 20, KH_2PO_4 7.0, Na_2HPO_4 2.5, $MgSO_4 \cdot 7H_2O$ 1.5, $FeCl_3 \cdot 6H_2O$ 0.15, $ZnSO_4 \cdot 7H_2O$ 0.02, $MnSO_4 \cdot H_2O$ 0.06, $CaCl_2 \cdot 2H_2O$ 0.15, $(NH_4)_2SO_4$ 1 and yeast extract 0.5 (Tchakouteu et al., 2015). The nitrogen concentration was maintained stable by addition of yeast extract at various time intervals in order to avoid

oleogenesis (Tapia et al., 2012). The culture was harvested by centrifugation at 4000 rpm for 10 min (moisture 26%).

The glucose media used for yeast immobilisation on DC as well as for the following cell growth/lipid accumulation experiments contained 12 and 40 g L^{-1} glucose, respectively.

Mixed AIW media were prepared by mixing orange juice with a diluted molasses solution of 4 ^{o}Be (Baume) hydrometer density, in order to prepare a cultivation broth containing 40 g L^{-1} total sugar. Orange juice was extracted from Washington Navel variety oranges (supplied from the local market). The oranges were blended for juice extraction and the solids were separated using a cheese cloth. The initial sugar concentration of the juice was 91 g L^{-1}. Various dilutions were then prepared using sterilised, deionized water. Molasses were supplied by the B. G. Spiliopoulos S. A. Distillery (Patras, Greece). They contained 78% solids, 42.5% total fermentable sugar and had pH 4.6. All media were sterilised by autoclaving for 15 min.

2.2 Preparation of DC and yeast immobilisation

The DC material was prepared after delignification of sawdust by boiling with 1% NaOH for 3 h. Cell immobilisation on DC took place by suspending 0.8 g of the harvested *L. starkeyi* cells in 80 mL of 12% glucose synthetic medium containing 1% peptone and 1% yeast extract, and mixing with 10 g of DC. The system was left to ferment for 6-8 h. The biocatalyst (DC with immobilised cells) was washed with fresh glucose medium. The immobilized yeast cells were estimated to be 0.2 g per 10 g of DC.

2.3 Cell growth and lipid accumulation in synthetic media

Amounts of 10.2 g of the immobilized biocatalyst were placed in 200-mL Erlenmeyer flasks, each containing 100 mL of 40 g L^{-1} glucose synthetic medium. The flasks were then agitated on an orbital shaker (P-Selecta Rotabit) at 180±5 rpm, and were incubated at various temperatures (28, 30, and 32 °C). The experiments were carried out with media having pH 4, 5 and 6, which was maintained stable by periodic additions of 5 M KOH. The cultivation broths were analysed at various time intervals for residual glucose. The same work was carried out with 0.2 g of suspended yeast cells (free cells). After consumption of all sugar, the free and immobilised cells were harvested by centrifugation to estimate the produced cell mass and lipid accumulation. All experiments were performed in triplicate and the average results are presented.

2.4 Cell growth and lipid accumulation in mixed AIW media

In the same manner, 10.2 g of immobilised biocatalyst or 0.2 g of free yeast cells were introduced in 200-mL flasks containing 100 mL of either 40 g L^{-1} glucose medium or mixed AIW substrate. The flasks were incubated at the optimum conditions obtained by the synthetic media experiments (30 °C and substrate pH 5). Again, the residual sugar was monitored (sum of sucrose, glucose and fructose), and after it was all consumed, the free or immobilised cells were harvested to estimate the produced cell mass and lipid accumulation. All experiments were also performed in triplicate.

2.5 Analytical methods

2.5.1 Sugar analysis by HPLC

Residual sugars at the end of each experiment were determined on a Shimadzu LC-9A HPLC system consisting of a Shim-pack SCR-101N column (set at 60 ^{o}C), an LC-9A pump, an RID-6A refractive index detector, a CTO-10A column oven, and a DGU-2A degassing unit. Ultra pure water, obtained by a Milli-Q water purifier system (resistivity 18.2 MΩ cm^{-1}), was used as mobile phase (0.8 mL/min). 1-Butanol (0.1% v/v) was used as internal standard. The sample dilution was 1% v/v, and the injection volume was 40 μL.

2.5.2 Cell mass determination

At the end of each cultivation experiment, the free or immobilized cells were harvested by centrifugation at 4500 rpm for 15 min and washed twice with distilled water. Cell mass production was determined by weighing after drying the cells at 80 ^{o}C overnight, and expressed as g dry cell mass per L of substrate (cell mass yield; g L^{-1}).

2.5.3 Lipid extraction

The intracellular lipid was extracted after homogenization of the cells with HCl and chloroform/methanol (2:1) (1 g of cells per 20 mL of solvent mixture), according to Folch et al. (1957). The whole mixture was agitated for 15-20 min on an orbital shaker and then allowed to stand in a dark place for 72 h. The extract was separated by paper filtration and evaporated on a rotary evaporator. This method allows extraction of the total lipid content, which was expressed as g lipid per g dry cell weight (SCO; g g^{-1} dry cell wt).

2.5.4 Fatty acid composition analysis

The microbial oil was esterified to produce methyl esters by addition of 230 mL of methanol/benzene (3:1) and careful dilution of 0.8 g of toluene sulphuric acid into the mixture. Methyl esters were determined on a GC-8A Shimadzu gas chromatograph connected with a C-R6A Chromatopack integrator, using a Free Fatty Acid Phase (FFAP) type stainless steel column suitable for esters (10% FFAP on Chromosorb W-AW 80/100, 3m long). The carrier gas was N_2 (20 mL/min). The injection port and the detector temperature was 250 ^{o}C. The column temperature range was 160-250 ^{o}C (6 ^{o}C/min rising rate). The internal standard was anthracene (0.7% w/v). Amounts of 2 μL of the samples were injected directly in the column. Identification of fatty acids was based on the retention times of the corresponding methyl esters.

3. Results and Discussion

Microbial SCO production is a promising alternative for exploitation of AIW for added-value products such as biodiesel and edible oils. However, application of such processes requires high productivities in order to maintain low production and investment costs. In this study, the growth and lipid accumulation by the oleaginous yeast *L. starkeyi* was evaluated using a mixed AIW substrate consisting of molasses and orange juice, two common food industry wastes (sugar production and citrus processing, respectively). The effect of yeast immobilization on a natural porous cellulosic material (DC) was also evaluated. The cultivation experiments were initially carried out in glucose media, at various temperature and pH conditions, using both free and immobilized cells. Subsequently, the mixed AIW substrate was used at the optimum conditions that were indicated by the experiments in the glucose media.

3.1 SCO production in glucose media

Cell growth and SCO accumulation by immobilised *L. starkeyi* was initially studied in glucose synthetic media, in shaken cultivation experiments and under limited nitrogen conditions, in order to identify the optimum temperature and pH conditions. The results are presented in Table 1. It can be observed that the mean SCO yield is significantly different (One-Way ANOVA analysis; P=0.003) at different substrate pH values. Regarding the temperature effect, the best results were obtained at 30 ^{o}C, although temperature in the range 28-32 ^{o}C does not seem to have a statistically significant effect (P=0.852) on SCO production. Therefore, substrate pH 5 and cultivation temperature 30 ^{o}C were selected as best conditions for lipid accumulation in free and immobilised yeast using AIW substrates. Similar cultivation conditions were also indicated as optimum in previous studies, regarding lipid accumulation in free *L. starkeyi* cells (Angerbauer et al., 2008; Lin et al., 2011; Zhao et al., 2008).

3.2 Effect of yeast immobilisation on SCO production

3.2.1 Cultivation in glucose media

SCO accumulation in *L. starkeyi,* free and immobilised on DC, was evaluated by cultivation experiments in glucose media (40 g L^{-1}). The process kinetics, regarding sugar consumption and lipid accumulation are shown in Figure 1. After 120 h cultivation of the immobilised cells, cell mass reached 22.5 g L^{-1} and SCO production was 0.33 g g^{-1} (Table 2), accounting for 8.8% and 40% increase, respectively, compared to free cells. Also, residual sugar was lower by 50.7% in the case of immobilised cells. This is in accordance with previous studies that showed better

assimilation of sugars (maltose, glucose, lactose) by cells immobilised on DC, which was explained by possible attraction of the sugars by hydrogen bonding on the DC surface and continuous pumping towards the cells (Ganatsios et al., 2014; Koutinas et al., 2012).

3.2.2 Cultivation in mixed AIW

Enhanced growth and SCO accumulation by the immobilised *L. starkeyi*, compared to free cells, was also observed in the mixed AIW substrate. Specifically, 17% higher cell mass production, 52% increase of SCO accumulation, and lower residual sugar were obtained (Table 2). These results are encouraging for efficient AIW exploitation for SCO production. Previous studies for exploitation of AIW substrates for SCO production reported lower cell mass and oil yields (Table 3). Therefore, cell immobilisation on natural low cost carriers such as DC, favours industrial opportunities for SCO production by renewable sources such as AIW.

3.2.3 Higher fatty acid composition of SCO

SCO applications in sectors such as edible oils and biofuels production, require suitable composition of fatty acids, regarding nutritional value and technological features. The lipids obtained by free or immobilised *L. starkeyi* cells were transesterified with methanol and then analyzed by GC. The analysis showed that the SCO glycerides contained saturated higher fatty acid (HFAs) such as palmitic and stearic acid, while unsaturated HFAs included oleic, palmitoleic and linoleic acids (Table 4). Yeast immobilisation on DC increased by 6% the unsaturated HFAs and reduced by 5% the saturated HFAs, indicating potential of the technique to improve the nutritional value of the oil. The 54% unsaturated HFAs content in the SCO

produced by immobilised *L. starkeyi*, as compared with soy oil, sunflower oil and corn oil, encourages research for the use of microbial SCO as edible oil.

3.3 Discussion

The promotional effect of the proposed immobilisation technique on *L. starkeyi* growth and lipid accumulation, was validated in both synthetic glucose media and in mixed AIW that contained orange juice and molasses. The yeast *L. starkeyi* was selected due to its ability to utilize various sugars, including glucose and xylose (Zhao et al., 2008), which are among the main sugars contained in wastes of the citrus and sugar industries as well as in other types of biomass.

Lipid accumulation is known to be significantly affected by the presence of specific metals in the substrate (e.g. Mg, K, Na, Fe, Zn, Co). It is also affected by limited nitrogen concentration that inhibits the activation of the NAD isocitrate dehydrogenase resulting in accumulation of citrate and increased acetyl-CoA and lipid synthesis (Tsakona et al., 2014; Shuib et al., 2014; Calvey at al., 2016). Therefore, the results of this study indicate that the used AIW contained sufficient metal and nitrogen sources for lipid accumulation in *L. starkeyi*.

Previous studies have also shown that cellulosic materials have an affinity for metal complexation (Kumar et al., 2017), which can lead to increased availability of the metal ions to the cells that are attached on DC. This, in combination with the naturally present metal ions in the mixed AIW, and the promotional effect of DC on the process, can lead to good lipid accumulation yields using the proposed renewable substrate and immobilisation technique.

Regarding the promotional effect of DC on bioprocessing, it has been previously

shown that its use as immobilization carrier can lead to increased productivities in alcoholic and lactic acid fermentations (Koutinas et al., 2012; Kourkoutas et al., 2004; Ganatsios et al., 2014). Specifically, in these studies, calculation of the activation energy of alcoholic fermentations at low temperatures showed that it was reduced by an average 42% when cells immobilized on DC were used.

Finally, the increased formation of oleic acid and the reduction of saturated HFAs are also of technological importance because they can contribute to the use of the produced SCO as edible oil. The proposed process is feasible, due to its low cost, simplicity, and environmentally friendly character.

4. Conclusions

The optimum conditions for growth and lipid accumulation of *L. starkeyi* cells immobilised on DC were 30 ^{o}C cultivation temperature and substrate pH 5. Comparison of immobilised and free cells in glucose media and in mixed AIW (molasses and orange juice) revealed the positive effect of the immobilisation technique on both growth and lipid accumulation. Immobilisation also increased the unsaturated HFAs and reduced the saturated HFAs content of the produced oil, leading to improvement of its nutritional value. The proposed process using renewable resources has potential for industrial application and further research is needed on SCO production for food uses.

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Figure captions

Figure 1. Glucose consumption and SCO accumulation by immobilised and free *L. starkeyi* cells in synthetic glucose media.

Table 1

Effect of pH and temperature on SCO production in glucose media (40 g L^{-1}) by *L. starkeyi* immobilized on DC.

Substrate pH	Cultivation temperature (^{o}C)	SCO (g g^{-1} dry cell wt)
6	32	0.19±0.07
	30	0.23±0.06
	28	0.18±0.10
5	32	0.24±0.07
	30	0.31±0.09
	28	0.28±0.07
4	32	0.12±0.05
	30	0.16±0.06
	28	0.10±0.05

Table 2

Cell mass yield and SCO production by free cells and immobilized *L. starkeyi* cells grown in glucose media (40 g L^{-1}) and in mixed AIW, at 30 ^{o}C and substrate pH 5.

	Cultivation time (h)	Cell mass yield (g L^{-1})	SCO (g g^{-1} dry cell wt)	Residual sugar* (g L^{-1})
Glucose media				
Free cells	120	20.5±0.82	0.23±0.03	1.96±0.94
Immobilized cells	120	22.5±0.91	0.33±0.05	1.30±0.65
AIW media				
Free cells	168	22.23±1.15	0.25±0.04	2.68±0.51
Immobilized cells	168	26±1.08	0.38±0.05	0.83±0.65

** Sum of residual sucrose, glucose and fructose in the fermented substrates*

Table 3

Comparison of the results of this study with those reported in the literature regarding the use of AIW for SCO production by the oleaginous yeast *L. starkeyi*.

Substrate	Initial sugar ($g\ L^{-1}$)	Sugar consumed ($g\ L^{-1}$)	Cell mass production ($g\ L^{-1}$)	SCO (% dry cell wt)	References
Free cells					
Potato peels	-	33.1	6.6	12.12	Ali El-Naggar et al., 2011
Cane molasses	-	38.3	7.4	16.22	Ali El-Naggar et al., 2011
Beet molasses	-	40.4	8.2	14.63	Ali El-Naggar et al., 2011
Tomato peels	-	35.6	5.4	11.11	Ali El-Naggar et al., 2011
Squash peels	-	29.9	4.9	12.24	Ali El-Naggar et al., 2011
Glucose syrup	-	35.6	8.5	16.47	Ali El-Naggar et al., 2011
Wheat straw hydrolysate	29.2-21.7 [a]	-	12.7-14.7	29.1-31.2	Yu et al., 2011
Fishmeal wastewater [b]	-	-	8.6	21.5	Huang et al., 2011
Corncob hydrolysate	42.3	-	17.2	47.0	Huang et al., 2014
Xylose + urea	60 + 1.03	-	94.6	37.4	Anschau and Franco, 2015
Cellobiose	70	-	27.9	50	Gong et al., 2012
Flour-rich waste hydrolysate	105	-	109.8	58.7	Tsakona et al., 2014
Monosodium glutamate wastewater [c]	-	-	4.6	24.7	Liu et al., 2012
Saccharified sweet sorghum stalks [d]			22	29.5	Matsakas et al., 2014
Cells immobilized on DC					
Mixed AIW	40		26	37	This study

a: sum of glucose, xylose, arabinose & galactose; b: supplemented with 20% glucose; c: supplemented with 80% glucose; d: with initial sorghum content 12%

Table 4

Fatty acid composition (%) of SCO produced by free and immobilised *L. starkeyi* grown in mixed AIW, at 30 ^{o}C and substrate pH 5.

	Fatty acids						
	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Saturated	Unsaturated
Free cells	36.0	2.5	12.0	45.0	3.5	48.0	51.0
Immobilized cells	34.0	2.7	11.5	49.0	2.4	45.5	54.1

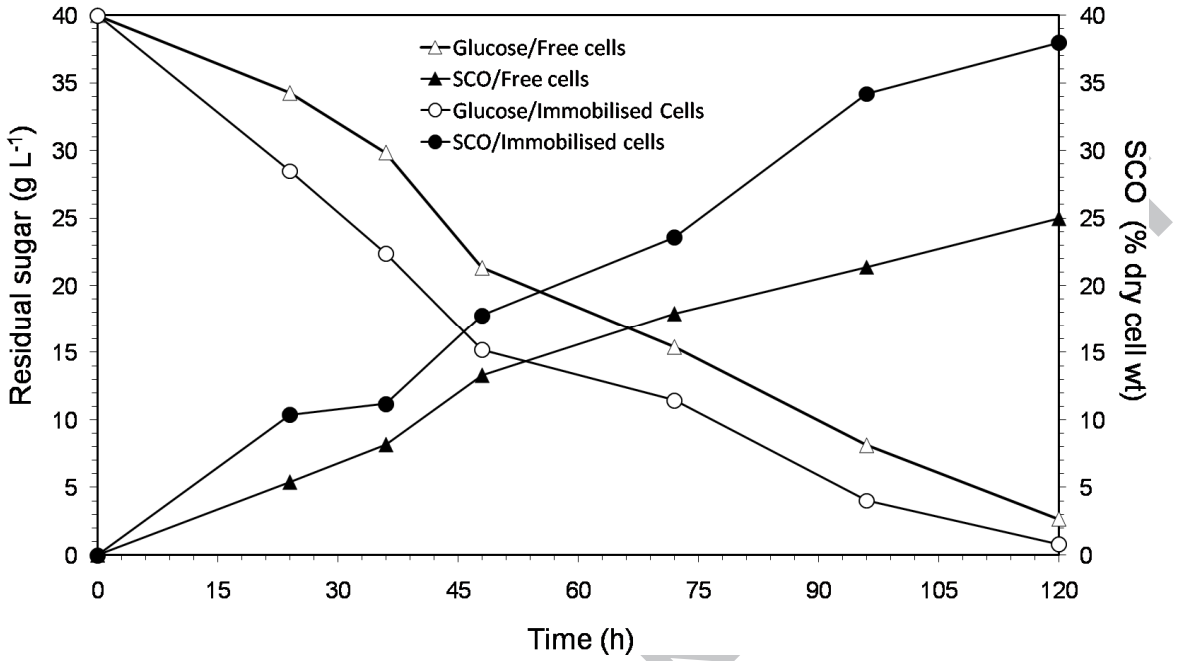

Fig. 1

Highlights

- Growth and SCO production by *L. starkeyi* immobilised on delignified cellulose
- Glucose and mixed AIW (molasses/orange juice) substrates were used
- 30 ^{o}C and pH 5 were the best conditions for both growth and SCO production
- 85% enhanced SCO production by the immobilized cells in the mixed AIW substrate
- 6% increase of unsaturated HFAs of the SCO produced by the immobilized cells